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Progression of Human Aortic Valve Stenosis Is Associated With Tenascin-C Expression

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OBJECTIVES We sought to assess tenascin-C (TN-C) expression and its possible pathobiological impact in human aortic valve stenosis.

BACKGROUND Tenascin-C, a large extracellular matrix glycoprotein, has lately been increasingly connected to cardiovascular pathologies. As TN-C is a multifunctional protein implicated in cell proliferation, migration and differentiation, we investigated the pattern of its expression in diseased human aortic valves.

METHODS Fifty-five tricuspid, non-rheumatic stenotic aortic valves were collected from patients undergoing aortic valve replacement, and the controls consisted of four normal valves from individuals who had suffered traumatic death and one from a patient operated on because of a noncalcified purely regurgitant valve. A monoclonal mouse antibody to human TN-C (143DB7) was used as the primary antibody in immunostaining. To study the source of TN-C messenger RNA synthesis, some tissue samples were also examined using in situ hybridization. In order to identify smooth muscle cell differentiation, commercially available antibodies against alpha-smooth muscle actin were used, and immunophenotypic analysis of inflammatory cells was carried out by using the monoclonal mouse antibodies UCHL-1, L26 and PGM-1.

RESULTS In normal valves, TN-C expression was associated with the basement membrane beneath the endothelial cells, whereas stenotic valves showed no such expression but rather immunoreactivity in the deeper layers of the valves. This reactivity was associated with the characteristics typical of the stenosing process and the increased mechanical loading caused by hypertension.

CONCLUSIONS We hypothesize that the overexpression of TN-C in stenotic human aortic valves may emphasize that this disease is an active rather than a degenerative process. (J Am Coll Cardiol 2002;39:96–101) © 2002 by the American College of Cardiology

The pathobiology of non-rheumatic aortic valve stenosis has changed over time, and recent observations have suggested that the development of the disease might be actively regulated and, thus, potentially modifiable, rather than being an inevitable consequence of aging (1). New evidence suggests that the molecular mechanisms and cellular components leading to valve calcification may resemble those of bone formation. Extracellular bone matrix proteins (osteopontin, osteocalcin and osteonectin) have been identified in diseased valves (2,3). Inflammatory cells, including macrophages and lymphocytes, are another distinct feature of valve mineralization (1). Recently, Mohler et al. (4) identified a population of valvular interstitial cells with osteoblast-like characteristics that spontaneously formed calcific nodules in cell culture.

Tenascin-C (TN-C) is a modular and multifunctional hexameric extracellular matrix (ECM) glycoprotein implicated in cell proliferation, migration, differentiation and apoptosis (5). Tenascin-C expression is less abundant in normal adult tissues but is induced in malignant tumors and during inflammation and tissue repair. Tenascin-C expression has also been increasingly documented in vascular diseases, and very recent evidence suggests that there may be a link between protein expression and the development of calcific aortic stenosis (6).

In the present study, we sought to determine the pattern of expression of TN-C in human non-rheumatic aortic valve stenosis. Tenascin expression was closely associated with the progression of the disease, and, based on TN-C's multifunctional properties, the results obtained give further confirmation that the stenosing process is more likely to be an active, rather than passive, process.

METHODS

Patients. Fifty-five tricuspid, non-rheumatic stenotic aortic valves were collected from patients (24 men and 31 women with a mean age of 71 years, range: 57 to 82 years) undergoing aortic valve replacement because of valvular stenosis. Some of the patients underwent surgery because of isolated valve stenosis, while some with milder valve pathology underwent a combined procedure, usually including coronary bypass grafting (18/55), which enabled us to obtain a relatively comprehensive range of disease conditions. The most frequent comorbidities were hypertension (30/55) and coronary artery disease (28/55). Five normal

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Abbreviations and Acronyms

cDNA	= complementary DNA
EC	= endothelial cell
ECM	= extracellular matrix
MMP	= matrixmetalloproteinase
mRNA	= messenger RNA
PCR	= polymerase chain reaction
TGF-beta	= transforming growth factor-beta
TN-C	= tenascin-C

valves were collected to serve as controls; four from individuals who had suffered traumatic death and one from a patient operated on because of a noncalcified, purely regurgitant valve. Macroscopic disease severity was classified as mild (n = 12) (opaque leaflets with focal areas of thickening and stiffness, mean transvalvular Doppler gradient <30 mm Hg), moderate (n = 23) (more distinct stiffness with emerging signs of calcification and clinically mild obstruction, mean gradient 30 to 70 mm Hg) or severe (n = 20) (definite areas of calcification, prominent cusp thickening and clinically pronounced obstruction, mean transvalvular gradient >70 mm Hg). Immediately after excision, the valves were fixed in formaldehyde and embedded in paraffin. Histologic and immunohistochemical analyses were made on the samples taken vertically through the leaflet near the central part.

Immunohistochemistry of TN-C. Sections 5 μm thick were deparaffinized in xylene and rehydrated in a graded series of alcohol dilutions. Endogenous peroxidase was consumed by treatment with 0.3% hydrogen peroxide in absolute methanol for 30 min. A monoclonal mouse antibody (Mab) to human tenascin (143DB7) was used as the primary antibody in TN-C immunostaining. The sections were incubated with the primary antibody (dilution 1:1000) at 4°C overnight, followed by biotinylated rabbit anti-mouse antibody (dilution 1:300) and ABC-complex (Dakopatts, Glostrup, Denmark). The color was developed with diaminobenzidine, whereafter the sections were mounted in an aqueous medium. The sections were counterstained with a light hematoxylin stain. In negative controls we substituted PBS (140 ml NaCl, 0.01 M phosphate buffer, pH 7.2) and normal mouse serum for the primary antibody.

TN-C in situ hybridization. A complementary DNA (cDNA) fragment (bases: 814 to 1,316) of full-length tenascin cDNA was synthesized by polymerase chain reaction (PCR) from a subclone HT-11 kindly provided by Dr. Luciano Zardi (Instituto Nazionale Per La Ricerca Sul Cancro, Genova, Italy) using the following primers:

5′ CCCTGCAGTGAGGAGCACGGCACA 3′,

5′ TGCCCATTGACACAGCGGCCATGG 3′.

The cDNA (503 base pair) obtained by PCR was subcloned into a TA vector (TA Cloning Kit, Invitrogen Inc., Groningen, The Netherlands). Sense and antisense RNA probes were generated from a linearized template by using a riboprobe transcription kit (Promega, Madison, Wisconsin), and the probes were labeled with ^{35}S-UTP (Amersham International, Buckinghamshire, United Kingdom) to a specific activity of more than 3×10^8. The hybridization, the post-hybridization washes and the detection of hybrids have been described previously (7).

Immunohistochemistry of cellular composition. In order to identify smooth muscle cell differentiation in the fibroblast type cells, commercially available antibodies against alpha-smooth muscle actin (Clone 1A4, Sigma Biosciences, St. Louis, Missouri) were used at a dilution of 1:1000. For an immunophenotypic analysis of inflammatory cells, monoclonal mouse antibodies UCHL-1, L26 and PGM-1 (Dako, Glostrup, Denmark) were carried out. The UCHL-1 antibody stains T-cells; L26 stains B-cells, and PGM-1 stains macrophages. All these antibodies were used at a dilution of 1:50.

We assessed the extent of inflammation, fibrosis and calcification in the valves as follows: the extent of inflammation was semiquantified as none, mild (+), moderate (++) or severe (+++) according to the number of inflammatory cells. The inflammatory infiltrate was considered mild if only a few scattered lymphocytes could be seen in valvular tissues, mainly in high-power fields. It was considered moderate if lymphocytes were easily detected in low-power fields. The lymphatic infiltrate was considered severe if the lymphocytes formed well-recognized groups of inflammatory cells in quantities exceeding 50 cells/HPF. Fibrosis and calcification were considered mild (+) if <25%, moderate (++) if 25% to 50% and severe (+++) if >50% of the valvular area contained fibrotic or calcific tissues.

Statistical analysis. SPSS 9.0 for Windows (Chicago, Illinois) was used for statistical analysis. The significances were determined by Fisher exact test. Significance was assumed at $p < 0.05$.

RESULTS

Histologic features of the valves. In undiseased valves, the pars fibrosa was dominated by dense and relatively well-organized collagen bundles. Within this collagen network, occasional elongated fibroblast-like cells were observed. The spongiosa layer consisted of loose, apparently collagen-poor connective tissue, which was slightly more cell-rich than the pars fibrosa. Stenotic valves, however, showed subendothelial thickening caused by a combination of interstitial fibrosis, an accumulation of basal calcified deposits and an infiltration of inflammatory cells, and the transmural disorganization of valve architecture was characterized by lipid accumulation, collagen fiber disarray and calcified nodules. In some cases between the endothelium and a calcium nodule, distinct heterotopic ossification was seen, and a distinctly visible cement line attaching the heterotopic bone to the calcium nodule was detected (Fig. 1A). Within valve tissue, numerous small vessels were seen, and this feature

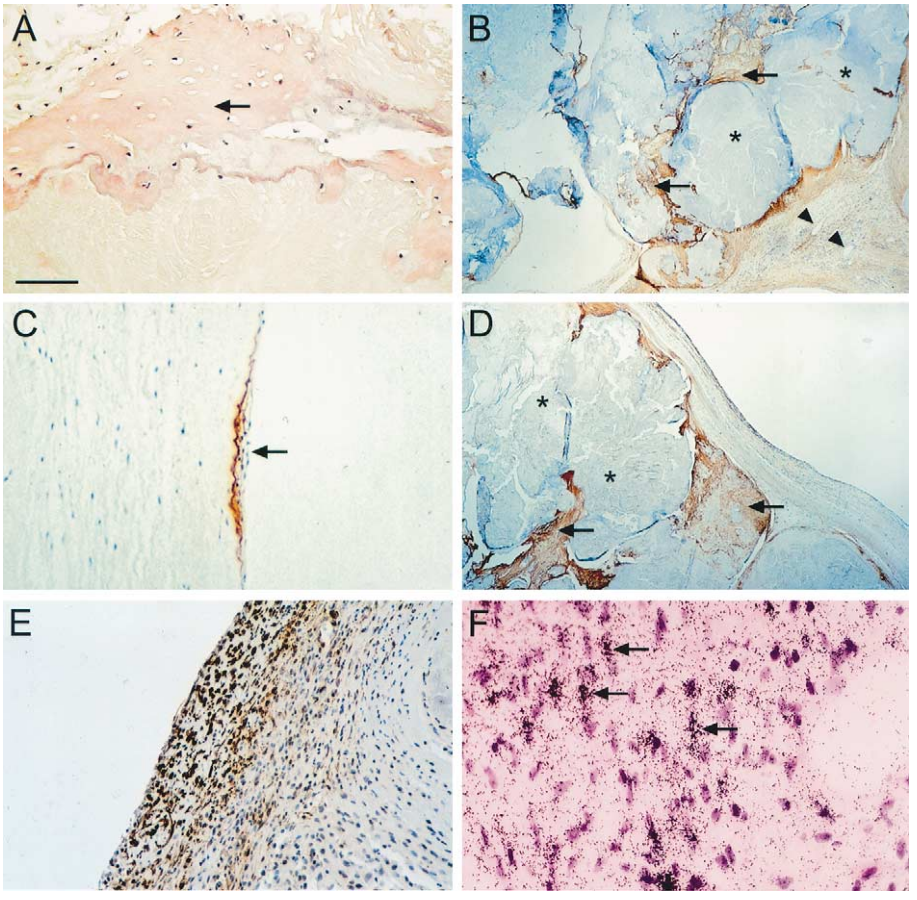

Figure 1. (A) A decalcified histologic section of an aortic valve stained with hematoxylin-eosin. Eosin-stained dystrophic bone is seen between the endothelial lining of the valve and the calcium nodule in the lower part of the image. A cement line joins the ossification to the calcium nodule. Dystrophic bone is marked with an **arrow.** Scale bar = 100 μm. **(B)** Intense tenascin-C (TN-C) immunostaining **(arrows)** can be seen in the vicinity of the calcified areas (marked with an **asterisk**) of the stenotic valve. Notice the inflammatory cells and the neovascularization **(arrowheads)** in the right corner of the field. **(C)** TN-C immunoreactivity can be seen as a basement membrane-associated zone in undiseased valves **(arrow).** The valvular stroma is otherwise negative. **(D)** Around the areas of calcification **(asterisk)** in a stenotic valve, intense TN-C immunopositivity **(arrows)** can be seen. Linear basement membrane-associated staining is missing. **(E)** Immunostaining with an antibody to alpha-smooth muscle actin. Strong positivity for this antigen can be seen in stromal fibroblast-type cells, suggesting myofibroblastic differentiation of the cells. **(F)** In situ hybridization of a stenotic valve. Strong signals for tenascin **(arrows)** can be seen in fusiform fibroblast-like cells in the stroma.

was more striking in the moderate-to-severe forms of valve pathology (Fig. 1B).

Immunohistochemistry of TN-C and cellular composition. Tenascin-C immunoreactivity (−/+/++/+++) was seen in undiseased valves as a basement-membrane-associated zone beneath the endothelial cells (EC) (Fig. 1C). In stenotic valves such expression was lacking, but a strong reaction was seen in the deeper parts of the valves, especially adjacent to the calcified areas (Fig. 1B and 1D).

In view of the degree of valve pathology, TN-C induction was more prominent in the group with severe alterations compared with the groups with mild or moderate changes ($p = 0.006$ and $p = 0.031$, respectively) (Fig. 2), and, accordingly, immunostaining was related to the extent of calcification, angiogenesis and fibrosis ($p = 0.031$, $p = 0.013$ and $p = 0.023$, respectively). Furthermore, expression was more abundant in hypertensive, rather than in normotensive, subjects ($p = 0.015$). Areas with pronounced inflammation tended to express more TN-C ($p = 0.07$). The majority of such inflammatory cells expressed a T phenotype and, to a lesser degree, a B phenotype. The association of T and B cells with TN-C immunoreactivity was suggestive ($p = 0.08$, $p = 0.06$, respectively). A similar tendency was observed with regard to the quantity of CD68-positive macrophages ($p = 0.06$). Some of the osteoclast-type cells observed in calcific nodules were also CD68-positive.

Fusiform alpha-smooth muscle actin-positive cells could be seen in all the cases studied. The number of these cells was frequently and profoundly increased in proportion to the degree of valvular pathology and TN-C expression (Fig. 1E).

TN-C in situ hybridization. In 12 selected cases, TN-C in situ hybridization experiments were performed. The messenger RNA (mRNA) signals for TN-C in the control valves were comparatively weak in most cases, but, in stenotic valves, increased TN-C mRNA expression was detected in individual stromal cells, whose positivity for alpha-smooth muscle actin suggested a myofibroblast phenotype (Fig. 1F).

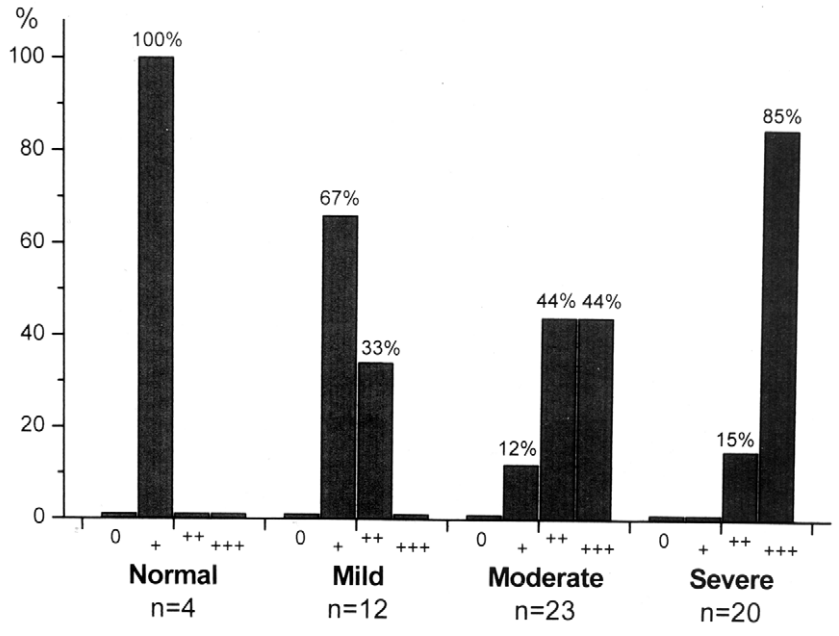

Figure 2. Tenascin-C expression presented in percentages in relation to the degree of valve pathology.

DISCUSSION

Major findings. This report provides with a comprehensive clinical series the first description of TN-C expression in human aortic valves. We found three novel features: 1) TN-C expression in normal valves was associated with the basement membrane beneath the ECs; 2) stenotic valves showed no such expression but showed immunoreactivity in the deeper layers of the valves, especially in calcific nodules; and 3) the positivity of TN-C staining in diseased cases was closely associated with the characteristics typical of the stenosing process. The main source of TN-C production in diseased valves seemed to be stromal myofibroblasts. In the control valves, TN-C mRNA signals were noticeably weak, which may suggest that the mRNA level required for maintaining a suitable protein concentration in tissues is low.

Potential mechanisms for TN-C upregulation. A number of factors may have led to the upregulation of TN-C expression observed here. For example, the basic fibroblast growth factor, which stimulates TN-C synthesis in other cell types, may be liberated in a bioactive form from the myofibroblast ECM stores by serine elastases (8). Given that increased serine elastase activity is observed in aortic valve lesions (6,9), it is possible that these enzymes induce TN-C expression. The stromal recruitment of mononuclear inflammatory cells and their suggestive association with TN-C possibly indicate that the soluble factors produced by these cells may stimulate protein expression, as interleukin-1beta has been shown to induce TN-C in a variety of cell types, including interstitial valvular cells (8). Further, the induction of a more homogenous pattern of expression of TN-C in progressive disease may be related to the paracrine induction of expression, which has been documented in vascular and other pathologies (10). This assumption is supported by the fact that interleukin-1 has been shown to play a role in the progression of pulmonary hypertension in monocrotaline-treated rats.

Since the increased expression of TN-C correlated temporally with the development and progression of the valvular lesions, especially under an increased hemodynamic loading, mechanical factors should also be considered. Mechanical forces are known to induce TN-C in neonatal rat cardiac myocytes in an amplitude-dependent manner (11). Chick embryo fibroblasts, vascular smooth muscle cells and whole pulmonary arteries cultured on attached (stretched) type I collagen substrates produce higher levels of TN-C than those cultivated on mechanically relaxed collagen gels (12,13). Similarly, the hemodynamic stress of increased arterial pressure induces TN-C expression in saphenous vein grafts interposed into arterial circulation (14). Mechanical pressure causes a redistribution of TN-C in scar tissue, and the increased pulmonary blood flow and the concomitantly increased strain exerted on vascular smooth muscle cells within the pulmonary arterial wall also lead to increased TN-C production in vivo (8). Collectively, the altered biomechanics may potentially account for the appearance of TN-C in tissues, where it is believed to play a regenerative or morphoregulatory role and may, therefore, elaborate the progressive lumen-to-media-directed protein induction detected in hypertensive patients with valve stenosis.

Pathobiological aspects on valvular stenosis. As regards its multifunctional properties, TN-C may have certain consequences for the pathogenesis of aortic valve stenosis. With respect to calcification, the present findings provided evidence that calcific nodules resembling heterotopic bone

were involved by TN-C. Cells of the osteoblast lineage are known to express TN-C from the onset of osteogenesis onwards, and TN-C can then modulate osteoblast behavior by stimulating cell differentiation and functional state (15). Tenascin-C, in transforming growth factor-beta-treated (TGF-beta) bone cells, is known to stimulate bone formation by mediating the osteogenic effects of TGF-beta in bone (16). The mechanical stress on TN-C and the calcification, the physical loading and the resulting increased strain imposed on rat ulnae lead to early increases in osteoblast TN-C expression, indicating that this protein may act as a mediator of osteoregulatory responses to altered biomechanics (17). Further, in vivo studies of TN-C expression in fibroblasts and chondrocytes within the osteotendinous junction under mechanical stress revealed a contemporaneous increase in both protein expression and ectopic ossification (18). Hence, we can postulate that TN-C may mediate the signal of increased hemodynamic stress as regards ossific alterations in valve tissue and that the calcific process is physiological, rather than degenerative, in nature.

Normal semilunar aortic valve cusps are sufficiently thin to allow complete nutrition by diffusion, and they are nearly avascular, although some investigators have described small blood vessels limited to the cuspal bases (19). In this study, diseased valves showed variable angiogenic changes, which were most obvious in moderate-to-severe forms of the disease and were significantly associated with TN-C expression. Angiogenesis is an important event in a variety of physiological settings, but it is also central to the etiology of a number of pathologic processes. Angiogenesis requires the breakdown and reassembly of the ECM, migration and proliferation of ECs and endothelial tube formation, and it is driven by a cocktail of growth factors and proangiogenic cytokines and tempered by an equally diverse group of inhibitors. In this regard matrixmetalloproteinases (MMP) have also received substantial attention. Type IV collagenase, also called MMP-2, can help degrade the subendothelial basement membrane starting the formation of a new channel. Jian et al. (6) in their recent work showed that TN-C has an important impact in the upregulation of both MMP-2 expression and its gelatinolytic activity. Interactions between cells and ECM molecules are mediated by cellular receptors, and TN-C is of particular interest in this connection because it binds to several integrins, including alpha2beta1 and alphavbeta3, both of which have been demonstrated to be involved in neovascularization (20). Furthermore, TN-C is known to be associated with the sprouting, but not the resting, phenotype of aortic ECs in vitro, and the angiogenic phenotype is inhibited when cells are grown in the presence of anti-TN-C antibodies, suggesting that the transition from a resting to a sprouting phenotype may be promoted by the reduced adhesive strength produced by TN-C (21).

Chronic inflammation was a common finding in diseased aortic valves, and the association was parallel to neovascularization and, to a lesser degree, TN-C expression. The importance of the inflammatory cellular infiltrate may lie in its potential modulation of endothelial and smooth muscle cell function through the cytokine network or even suggest a possible immune-mediated mechanism in valvular pathology. The specific factors that initially attract macrophages and lymphocytes into the valvular stroma remain unknown. Our results may provide one possible explanation for the mechanics of cellular accumulation, emphasizing the adhesive function of TN-C. Previous static binding assays have not confirmed the assumption that peripheral blood cells adhere to this glycoprotein (5). Two recent studies, however, have largely settled this matter and explained conclusively the modulation of interaction between lymphocytes and TN-C, providing further overall characterization of the adhesive properties of TN-C. First, Gundersen et al. (22) reported their novel finding that stimulated T lymphocytes can induce degradation of the ECM protein TN-C by plasminogen-dependent proteolysis and that this degradation acts as a feedback mechanism to selectively modulate the adhesive interaction between T lymphocytes and TN-C. This observation lent support to the presumption that the physical integrity of the TN-C molecule may determine its adhesive or antiadhesive activity and that limited proteolysis may provide a physiological mechanism mediating this activity. Contemporaneously with the work of Gundersen, Clark et al. (23) further pointed out that, under experimental shear flow conditions, peripheral blood leukocytes and several cell lines, especially of the T-cell lineage, may show low-avidity and transient adhesion to TN-C, which results in tethering and rolling. Taken together, the obvious association of inflammatory cells and TN-C with microcapillaries in the stroma of stenotic valves may result in adhesion, with inflammatory cells rolling along the endothelial surface, and then tight adherence of cells, resulting in immobilization and transmigration across the damaged endothelium into the inner layers via a route provided by these small vessels.

Summary. We propose that the increased expression of TN-C in stenotic aortic valves emphasizes the possibility that, rather than being a degenerative process, aortic valve mineralization is an active process. Models created in vitro to manipulate TN-C induction by, for example, using monoclonal antibodies against human TN-C may serve as future therapies to prevent and treat calcific valvular disease based upon the interfering role of TN-C in the mineralization mechanism.

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